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(54) Title: METHODS FOR DETECTION OF HUMAN PAPILLOMAVIRUS MRNA

(57) Abstract: Methods for detecting the presence of mRNA transcripts from the E6 or E7 genes of human papillomavirus in clinical samples using nucleic acid sequence-based amplification. Sets of degenerate oligonucleotide primers which may be used for simultaneous detection of multiple HPV types are also described.

METHODS FOR DETECTION OF HUMAN PAPILLOMAVIRUS mRNA

Field of the invention

The present invention is concerned with methods of detecting the presence of human papillomavirus mRNA in clinical samples using nucleic acid sequence-based amplification and in particular with sets of degenerate oligonucleotide primers for use in such methods.

Background to the invention

Cervical cancer is closely associated with human papillomavirus (HPV). More than 100 HPV genotypes have been considered to date; types 16, 18, 31, 33, 35, 45, 52, 58 and 67 are considered to belong to the group that puts patients infected with these types at high risk for cervical carcinogenesis. Specific viral genes (E6 and E7) from HPV types 16, 18 and 33 have been proved to act as oncogenes. Their expression seems to be necessary, but not sufficient, for conversion to malignancy. Absence of HPV Type 16 E6 transcripts in HPV 16 infected, cytological normal cervical scraping prove that E6 and E7 markers may be used as prognostic markers for screening of women at risk for the development of cervical carinoma (Falcinelli et al., J. Med. Virology., 1993). With in situ hybridisation an increase in the expression of the viral early region was observed with increasing cellular differentiation and in high-grade lesions a uniform ditribution of high expression was observed (Durst et al., Virology, 189:132-140., 1992). E7 protein is multifunctional and enhances the degradation of retinoblastoma gene products (Boyer et al., 1996) and the E6 proteins accelerate in vitro degradation of P53 protein and effect the stability of p53 protein intracellularly (Lechner et al., 1992). The E6 and E7 proteins are oncogenic, not only because of the

degradation of p53, RB and other tumour suppressor proteins, but also because of their influence on cell cycle control mediators p21, p27, E2F (transcription factor) and PCNA (proliferating cell nuclear antigen). HPV types 16, 18 and 33 are often found integrated into chromosomes of cancerous cervical cells, and this integration has been postulated to result in increased levels of stabilities of gene E6 and E7 expression. Jeon and coworkers have also demonstrated that HPV type 16 DNA integration correlates with increased levels of expression of the viral E7 protein and with a selective growth advantage over cells harbouring extrachromosomal HPV16 DNA. The existence of HPV-negative cervical cancers is now debatable; zur Hausen and de Villiers have argued that if they do exist, their incidence should be low.

In the last few years, there has been an improvement in the methods used to detect HPV, with methods based on amplification of nucleic acids using the polymerase chain reaction (PCR) becoming increasingly widespread. It is now possible to detect small amounts of HPV DNA (<100 pg), quantify the amount of viral DNA in clinical samples, identify a broad spectrum of genital HPV types, test for selected HPV types and localise the viral genome transcripts and proteins to the individual cells. Since HPV detection is often carried out in the presence of vast quantities of host nucleic acids and cells not infected with the virus, the ability of the primers to be virus specific is critical for a sensitive and specific amplification.

Concern has been expressed about the reproducibility of the PCR technique alone following reports of widely different estimates of the prevalence of HPV 16 DNA sequences in normal and abnormal cervical epithelial (Franco et al., 1991). These problems of reproducibility have also been a

problem in squamous cell cervical carcinoma series where the detection rate of HPV 16 varied from 45 to 87%. This may be due to the sample preparation differences, inadequate primer construction and other methodological differences. However, one important factor is to include a probe related detection of the amplified product in order to increase the sensitivity.

NASBA (Nucleic acid sequence-based amplification) is a relatively new method for the amplification of RNA (Compton, Nature. 350: 91-92 (1991)). NASBA is well known by persons of ordinary skill in the art and is described, for example, in US-A-5,409,818. NASBA is an effective procedure for generating large quantities of a target RNA sequence *in vitro*, allowing detection of target RNA sequences that are present in very low concentrations in the original test sample.

The NASBA method is based on the same type of primer-sets as for PCR but one primer is modified with a promotor sequence, for example a T7 promoter. The sensitivity and specificity of the NASBA amplification has been shown to be the same as for PCR and better then most RT-PCR protocols. Since the NASBA method is an isothermal assay and it is dependent on RNase H it cannot amplify DNA. This is important in connection with detection of mRNA expression from the HPV genome where the RT-PCR method would amplify both the RNA and DNA. There is extensive literature on the use of NASBA for the detection of HIV-1 in clinical samples (see for example Kievits et al., Journal of Virological Methods. 35: 273-286 (1991)).

The present inventors have now applied the NASBA technique to the detection of human papillomavirus (HPV) in test samples. The inventors' approach is based upon the development of degenerate primer

cocktails which allow simultaneous detection of two or more HPV types.

Description of the invention

In a first aspect the invention provides oligonucleotides, more particularly degenerate oligonucleotides, which may be used for the detection of specific combinations of HPV types.

In a first embodiment the invention provides an oligonucleotide for use in detecting the presence of human papilloma virus type 16, type 31,type 35 or a combination thereof in a test sample, said oligonucleotide being selected from the group consisting of:

oligonucleotides comprising one of the following nucleotide sequences:

Primer 1 oligonucleotides:
5' X_1-AGRTCAGTTGYCTCDGGT [SEQ ID NO:1]
5' X_1-TCYGGTTYTGCTTGTCCA [SEQ ID NO:2]
5' X_1-TGTGTGCTYTGTACRCACAR [SEQ ID NO:3]
wherein X_1 represents a nucleotide sequence comprising a promoter,

Primer 2 oligonucleotides:
5' CCRYTGTGTCCWGWWGAA [SEQ ID NO:4]
5' TGCRTGGAGAWAYAMCTA [SEQ ID NO:5]
5' TATRTKTTAGATTTGSAACC [SEQ ID NO:6]

Probe oligonucleotides:
5' TGTAAWCATGCRTGGAGAWA [SEQ ID NO:7]
5' GAAACCSARSTGTAAWCATG [SEQ ID NO:8]
5' CATGCRTGGAGAWAYAMCTA [SEQ ID NO:9]
5' GACAGCTCAGAKGAGGAGGA [SEQ ID NO:10]
5' CAACTGAYCTMYACTGTTATGA [SEQ ID NO:11]

5' CAACTGAYCTMYACTGTTATGAGCAATT [SEQ ID NO:12]

and oligonucleotides comprising one of the following structures:

'Molecular beacon' probes:

5'X_2-CCGAGCACTGAYCTMYACTGTTATGAGCTCGG-X_3 [SEQ ID NO:13]

5'X_2-CCAAGCACTGAYCTMYACTGTTATGAGCTTGG-X_3 [SEQ ID NO:14]

5'X_2-CCAAGCCAACTGAYCTMYACTGTTATGAGCAGCTTGG-X_3 [SEQ ID NO:15]

wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

In a second embodiment the invention provides an oligonucleotide for use in detecting the presence of human papilloma virus type 52, type 58, type 67 or a combination thereof in a test sample, said oligonucleotide being selected from the group consisting of:

oligonucleotides comprising one of the following nucleotide sequences:

Primer 1 oligonucleotides:

5' X_1-TCCTCRTCTGAGCTGTCA [SEQ ID NO:16]

5' X_1-TGCTTGTCCATCTGGCCGGT [SEQ ID NO:17]

wherein X_1 represents a nucleotide sequence comprising a promoter,

Primer 2 oligonucleotides:

5' TGGACAGGRCGSTGTKCA [SEQ ID NO:18]

5' TGTKCAGWGTGTTGGAGA [SEQ ID NO:19]

Probe oligonucleotides:

5' CAACTGACCTAYWCTGCTATGA [SEQ ID NO:20]

5' GAAMCAACTGACCTAYWCTGCTAT [SEQ ID NO:21]

and oligonucleotides having one of the following structures:

'Molecular beacon' probes:

5' X_2-CCAAGCAANCAACTGACCTAYWCTGCTAGCTTGG-X_3 [SEQ ID NO:22]

5' X_2-CCAAGCACTGACCTAYWCTGCTATGAGCTTGG-X_3 [SEQ ID NO:23]

wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

In a third embodiment the invention provides an oligonucleotide for use in detecting the presence of human papilloma virus type 33, type 58 or a combination thereof in a test sample, said oligonucleotide being selected from the group consisting of:

oligonucleotides comprising one of the following nucleotide sequences:

Primer 1 oligonucleotides:

5' X_1-CAAGTGTRACAACARGTTA [SEQ ID NO:24]

5' X_1-GCACAGSTAGGGCACACAA [SEQ ID NO:25]

wherein X_1 represents a nucleotide sequence comprising a promoter,

Primer 2 oligonucleotides:

5' CAACTGACCTATWCTGCTA [SEQ ID NO:26]

5' ATCCTGAACCAACTGACCTA [SEQ ID NO:27]

Probe oligonucleotides:

5'GTGACAGCTCAGAYGAGGATGA [SEQ ID NO:28]

5'GGCCAGATGGACAAGCACAAC [SEQ ID NO:29]

and oligonucleotides comprising one of the following structures:

'Molecular beacon' probes:

5' X_2-CCAAGCGCCAGATGGACAAGCACAAGCTTGG-X_3 [SEQ ID NO:30]

5' X_2-CCGAGCGCCAGATGGACAAGCACAAGCTCGG-X_3 [SEQ ID NO:31]

wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

In a fourth embodiment the invention provides an oligonucleotide for use in detecting the presence of human papilloma virus type 18, type 45 or a combination thereof in a test sample, said oligonucleotide being selected from the group consisting of:

oligonucleotides comprising one of the following nucleotide sequences:

Primer 1 oligonucleotides:

5' X_1-AGCTCAATTCTGSCKTCA [SEQ ID NO:32]

5' X_1-ACGGACACACAAAGGACA [SEQ ID NO:33]

5' X_1-GCACACCACGGACACACA [SEQ ID NO:34]

wherein X_1 represents a nucleotide sequence comprising a promoter,

Primer 2 oligonucleotides:

5' ACGAGCAATTAAGCGAST [SEQ ID NO:35]

5' CCGACGAGCCGAACCACA [SEQ ID NO:36]

Probe oligonucleotides:

5'AGCCCGACGAGCCGAACCACA [SEQ ID NO:37]

5'GTTGTAAGTGTGAMGSCAGAATT [SEQ ID NO:38]

and oligonucleotides having the following structure:

5' X_2-CCAAGCAGCCCGACGAGCCGAACCACAGCTTGG-X_3 [SEQ ID NO:39]

wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

The oligonucleotide molecules of the invention are preferably single stranded DNA molecules. Non-natural synthetic polynucleotides which retain the ability to base-pair with a complementary nucleic acid molecule and are able to function in the NASBA reaction are also within the scope of the invention, including synthetic oligonucleotides which incorporate modified bases and synthetic oligonucleotides wherein the links between individual nucleosides include bonds other than phosphodiester bonds. The oligonucleotide molecules of the invention may be produced according to techniques well known in the art, such as by chemical synthesis using standard apparatus and protocols for oligonucleotide synthesis. Representations of the sequences of degenerate oligonucleotide molecules provided herein use the standard IUB code for mixed base sites:

N=G,A,T,C; V=G,A,C; B=G,T,C; H=A,T,C; D=G,A,T; K=G,T; S=G,C;W=A,T; M=A,C; Y=C,T; R=A,G.

The oligonucleotide molecules of the invention are selected to be specific for mRNA transcribed from the HPV E6 or E7 genes, expression of which is associated with cervical cytological abnormalities which often progress to more serious disease. A number of studies relate the expression of the E6 and E7 genes to oncogenesis. Co-operation between E6 and E7 increases significantly the frequency of immortalization. Evidence has been presented that the

E6 and E7 open reading frames are involved in the transforming activity of the virus (Tanaka et al., J. Virol. 63: 1465-1469, 1989). These transformation effects of E6 and E7 may at least in part be explained by their interaction with the cellular tumour suppressor gene products p53 and pRb 105, respectively (Boyer et al., Cancer Research. 56: 4620-4624, 1996; Lechner et al. EMBO J. 11: 3045-3051, 1992). There is an extraordinary conservation of the E6 and E7 DNA and RNA sequence (Cone et al. J Med Virol. 37: 99-107. 1992). A number of publications have reported that E6 and E7 sequences are specific for each subtype of HPV (Karlsen et al., Eur J Cancer. 31A: 1511-1516, 1995; Karlsen et al., J Clin Microbiol. 34: 2095-2100, 1996).

The oligonucleotide molecules provided by the invention fall into four groups. Primer 1 (P1) oligonucleotide molecules are generally approximately 50 bases in length, with an average of about 20 bases at the 3' end that are complementary to a region of the target HPV mRNA. One or more of the bases in the 3' end region are degenerate, and this degeneracy results in specificity for two or more HPV types. The 5' ends of the primer 1 oligonucleotides (represented herein in general terms as X_1) comprise a promoter sequence that is recognized by a specific RNA polymerase. Bacteriophage promoters, for example the T7, T3 and SP6 promoters, are preferred for use in the oligonucleotides of the invention, since they provide advantages of high level transcription which is dependent only on binding of the appropriate RNA polymerase. In a preferred embodiment, the 5' terminal sequence of the primer 1 oligonucleotides may comprise the sequence AATTCTAATACGACTCACTATAGGG [SEQ ID NO:40] or the sequence AATTCTAATACGACTCACTATAGGGAGAAGG [SEQ ID NO:62]. These

sequences contain a T7 promoter, including the transcription initiation site for T7 RNA polymerase.

The primer 2 (P2) oligonucleotides generally comprise a sequence of approximately 20 bases substantially identical to a region of the target HPV mRNA. As with the primer 1 oligonucleotides one or more of the bases in this sequence are degenerate, resulting in specificity for two or more HPV types. The primer 2 oligonucleotides may, in a particular but non-limiting embodiment, further comprise a sequence of nucleotides at the 5' end which is unrelated to HPV but which is capable of hybridising to a generic detection probe. The detection probe will preferably be labelled, for example with a fluorescent, luminescent or enzymatic label. In one embodiment the detection probe is labelled with a label that permits detection using ECL™ technology, although it will be appreciated that the invention is in no way limited to this particular method of detection. In a preferred embodiment the 5' end of the primer 2 oligonucleotides may comprise the sequence GATGCAAGGTCGCATATGAG [SEQ ID NO:41]. This sequence is capable of hybridising to a generic ECL™ probe commercially available from Organon Teknika having the following structure:

$Ru(bpy)_3^{2+}$-GAT GCA AGG TCG CAT ATG AG-3'

In a different embodiment the primer 2 oligonucleotide may incorporate 'molecular beacons' technology, which is known in the art and described, for example, in WO 95/13399 by Tyagi and Kramer, Nature Biotechnology. 14: 303-308, 1996, to allow for real-time monitoring of the NASBA reaction.

The third type of oligonucleotide molecules provided by the invention are HPV-specific probe oligonucleotides. The probe oligonucleotides generally comprise a sequence of approximately 20-25

bases substantially identical to a region of the target HPV mRNA. One or more of the bases in this sequence may be degenerate, resulting in a probe mix which has specificity for two or more HPV types. The probe oligonucleotides may be used as HPV-specific hybridisation probes for detection of the products of a NASBA reaction. In this connection the probe oligonucleotides may be coupled to a solid support, such as paramagnetic beads, to form a capture probe (see below). In a preferred embodiment the 5' end of the probe oligonucleotide may be labelled with biotin. The addition of a biotin label facilitates attachment of the probe to a solid support via a biotin/streptavidin or biotin/avidin linkage.

The fourth type of oligonucleotide molecules provided by the invention are HPV-specific probes incorporating 'molecular beacons' technology which is known in the art and described, for example, by Tyagi and Kramer, Nature Biotechnology. 14: 303-308, 1996 and in WO 95/13399. The use of molecular beacons technology allows for real-time monitoring of the NASBA reaction. The molecular beacons primers generally include, in addition to a HPV-specific sequence including one or more degenerate bases, additional bases which allow formation of a hairpin loop structure, a fluorescent moiety and a quencher moiety, the fluorescent and the quencher moieties being represented herein by the notation X_2 and X_3. As will be appreciated be the skilled reader, the fluorescer and quencher moieties are selected such that the quencher moiety is capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two moieties are in close proximity, e.g. when the probe is in the hairpin 'closed' conformation in the absence of the target sequence. Many examples of suitable pairs of quencher/fluorescer moieties which may be used in

accordance with the invention are known in the art (see WO 95/13399, Tyagi and Kramer, ibid). Preferred combinations include the fluorophore 5-(2'-aminoethyl)aminonaphthalene-1-sulphonic acid (EDANS) and the quencher 4-(4'-dimethylaminophenylazo)benzoic acid (DABCYL), although it will be appreciated that the invention is not limited to this particular combination. The fluorescer and quencher moieties may be covalently attached to the probe in either orientation, either with the fluorescer at or near the 5' end and the quencher at or near the 3' end or vice versa.

The oligonucleotide molecules of the invention may be used in the detection of HPV mRNA by NASBA. In particular, suitable combinations of primer 1 and primer 2 oligonucleotide molecules may be used to drive a NASBA amplification reaction. In order to drive a NASBA amplification reaction the primer 1 and primer 2 oligonucleotides must be capable of priming synthesis of a double-stranded DNA from a target region of mRNA, specifically HPV E6-E7 mRNA. For this to occur the primer 1 and primer 2 oligonucleotides must comprise HPV-specific sequences which are complementary to regions of the sense and the antisense strand of the target mRNA, respectively.

In the first phase of the NASBA amplification cycle, the so-called 'non-cyclic' phase, the primer 1 oligonucleotide anneals to a complementary sequence in the target mRNA and its 3' end is extended by the action of an RNA-dependent DNA polymerase (e.g. reverse transcriptase) to form a first-strand cDNA synthesis. The RNA strand of the resulting RNA:DNA hybrid is then digested, e.g. by the action of RnaseH, to leave a single stranded DNA. The primer 2 oligonucleotide anneals to a complementary sequence towards the 3' end of this single stranded DNA and its

3' end is extended (by the action of reverse transcriptase), forming a double stranded DNA. RNA polymerase is then able to transcribe multiple RNA copies from the now transcriptionally active promoter sequence within the double-stranded DNA. This RNA transcript, which is antisense to the original target mRNA, can act as a template for a further round of NASBA reactions, with primer 2 annealing to the RNA and priming synthesis of the first cDNA strand and primer 1 priming synthesis of the second cDNA strand. The general principles of the NASBA reaction are well known in the art (see Compton, J. Nature. 350: 91-92).

The HPV-specific probe oligonucleotides described herein may also be attached to a solid support, such as magnetic microbeads, and used as 'capture probes' to immobilise the product of the NASBA amplification reaction (a single stranded RNA). The HPV-specific 'molecular beacons' probes described herein may be used for real-time monitoring of the NASBA reaction (see Leone *et al.*, Nucleic Acids Research., 1998, vol: 26, pp 2150-2155).

A list of specific oligonucleotide primers and probes provided by the invention is given below. However, it is not intended that this list should in any way be interpreted as limiting the scope of the invention to these specific molecules:

Oligonucleotides for the detection of HPV 16, 31, 35:

Primer 1 oligonucleotides

HPV AP2-1	5'AATTCTAATACGACTCACTATAGGGAGAAGGA GRTCAGTTGYCTCDGGT [SEQ ID NO:42]
HPV AP1-1	5'AATTCTAATACGACTCACTATAGGGAGAAGGT CYGGTTYTGCTTGTCCA [SEQ ID NO:43]
HPV AP3-1	5'AATTCTAATACGACTCACTATAGGGAGAAGGT GTGTGCTYTGTACRCACAR [SEQ ID NO:44]

Primer 2 oligonucleotides:

HPV AP2-2 5'GATGCAAGGTCGCATATGAGCCRYTGTGTCCW GWWGAA [SEQ ID NO:45]

HPV AP1-2 5'GATGCAAGGTCGCATATGAGTGCRTGGAGAWA YAMCTA [SEQ ID NO:46]

HPV AP3-2 5'GATGCAAGGTCGCATATGAGTATRTKTTAGAT TTGSAACC [SEQ ID NO:47]

Probe oligonucleotides:

HPV APO2A 5'TGTAAWCATGCRTGGAGAWA [SEQ ID NO:7]

HPV APO2B 5'GAAACCSARSTGTAAWCATG [SEQ ID NO:8]

HPV APO2 5'CATGCRTGGAGAWAYAMCTA [SEQ ID NO:9]

HPV APO1 5'GACAGCTCAGAKGAGGAGGA [SEQ ID NO:10]

HPV APO1A 5'CAACTGAYCTMYACTGTTATGA [SEQ ID NO:11]

HPV APO1B 5'CAACTGAYCTMYACTGTTATGAGCAATT [SEQ ID NO:12]

Oligonucleotides for the detection of HPV 52, 58, 67:

Primer 1 oligonucleotides:

HPV BP2-1 5'AATTCTAATACGACTCACTATAGGGAGAAGGT CCTCRTCTGAGCTGTCA [SEQ ID NO:48]

HPV BP2A-1 5'AATTCTAATACGACTCACTATAGGGAGAAGGT GCTTGTCCATCTGGCCGGT [SEQ ID NO:49]

Primer 2 oligonucleotides

HPV BP2-2 5'GATGCAAGGTCGCATATGAGTGGACAGGRCGS TGTKCA [SEQ ID NO:50]

HPV BP2A-2 5'GATGCAAGGTCGCATATGAGTGTKCAGWGTGT TGGAGA [SEQ ID NO:51]

Probe oligonucleotides:

HPV BPO2 5'CAACTGACCTAYWCTGCTATGA [SEQ ID NO:20]

HPV BPO2A 5'GAAMCAACTGACCTAYWCTGCTAT [SEQ ID NO:21]

Oligonucleotides for the detection of HPV 33, 58:

Primer 1 oligonucleotides:

HPV BP1-1 5'AATTCTAATACGACTCACTATAGGGAGAAGGC AAGTGTRACAACARGTTA [SEQ ID NO:52]

HPV BP1A-1 5'AATTCTAATACGACTCACTATAGGGAGAAGGG CACAGSTAGGGCACACAA [SEQ ID NO:53]

Primer 2 oligonucleotides:

HPV BP1-2 5'GATGCAAGGTCGCATATGAGCAACTGACCTAT WCTGCTA [SEQ ID NO:54]

HPV BP1A-2 5'GATGCAAGGTCGCATATGAGATCCTGAACCAA CTGACCTA [SEQ ID NO:55]

Probe oligonucleotides:

HPV BPO1 5'GTGACAGCTCAGAYGAGGATGA [SEQ ID NO:28]

HPV BPO1A 5'GGCCAGATGGACAAGCACAAC [SEQ ID NO:29]

Oligonucleotides for the detection of HPV 18,45:

Primer 1 oligonucleotides:

HPVCP1-1 5'AATTCTAATACGACTCACTATAGGGAGAAGGA GCTCAATTCTGSCKTCA [SEQ ID NO:56]

HPVCP2-1 5'AATTCTAATACGACTCACTATAGGGAGAAGGA CGGACACACAAAGGACA [SEQ ID NO:57]

HPVCP3-1 5'AATTCTAATACGACTCACTATAGGGAGAAGGG CACACCACGGACACACA [SEQ ID NO:58]

Primer 2 oligonucleotides:

HPVCP1-2 5'GATGCAAGGTCGCATATGAGACGAGCAATTAA GCGAST [SEQ ID NO:59]

HPVCP2-2 5'GATGCAAGGTCGCATATGAGACGAGCAATTAA GCGAST [SEQ ID NO:60]

HPVCP3-2 5'GATGCAAGGTCGCATATGAGCCGACGAGCCGA ACCACA [SEQ ID NO:61]

Probe oligonucleotides:

HPVCPO1 5'AGCCCGACGAGCCGAACCACA [SEQ ID NO:37]

HPVCPO3 5'GTTGTAAGTGTGAMGSCAGAATT [SEQ ID NO:38]

Cocktails of primer-sets and probes comprising certain combinations of the oligonucleotide molecules of the invention are also the subject of the present invention, these primer cocktails being useful for the detection of various combinations of two or more HPV types by NASBA.

Therefore, in a particular embodiment the invention provides a first primer cocktail which is suitable for use in detecting the presence of human papilloma virus type 16, type 31, type 35 or a combination thereof in a test sample, the primer cocktail comprising a combination of oligonucleotide molecules having the following nucleotide sequences:

5' CCRYTGTGTCCWGWWGAA [SEQ ID NO:4]
5' TGCRTGGAGAWAYAMCTA [SEQ ID NO:5]
5' TATRTKTTAGATTTGSAACC [SEQ ID NO:6]
5' X_1-AGRTCAGTTGYCTCDGGT [SEQ ID NO:1]
5' X_1-TCYGGTTYTGCTTGTCCA [SEQ ID NO:2]
5' X_1-TGTGTGCTYTGTACRCACAR [SEQ ID NO:3]

wherein X_1 represents a nucleotide sequence comprising a promoter,

In a preferred embodiment this first primer cocktail comprises the following combination of oligonucleotide molecules:

Primer name	Sequence
HPV AP2-1	5'AATTCTAATACGACTCACTATAGGGAGAAGGA GRTCAGTTGYCTCDGGT [SEQ ID NO:42]
HPV AP2-2	5'GATGCAAGGTCGCATATGAGCCRYTGTGTCCW GWWGAA [SEQ ID NO:45]
HPV AP1-1	5'AATTCTAATACGACTCACTATAGGGAGAAGGT CYGGTTYTGCTTGTCCA [SEQ ID NO:43]
HPV AP1-2	5'GATGCAAGGTCGCATATGAGTGCRTGGAGAWA YAMCTA [SEQ ID NO:46]
HPV AP3-1	5'AATTCTAATACGACTCACTATAGGGAGAAGGT

GTGTGCTYTGTACRCACAR [SEQ ID NO:44]

HPV AP3-2 5'GATGCAAGGTCGCATATGAGTATRTKTTAGAT TTGSAACC [SEQ ID NO:47]

In a further embodiment the invention also provides a primer cocktail/probe mixture comprising the first primer cocktail according to the invention and a mixture of probe oligonucleotide molecules having the following nucleotide sequences:

5' TGTAAWCATGCRTGGAGAWA [SEQ ID NO:7]
5' GAAACCSARSTGTAAWCATG [SEQ ID NO:8]
5' CATGCRTGGAGAWAYAMCTA [SEQ ID NO:9]
5' GACAGCTCAGAKGAGGAGGA [SEQ ID NO:10]
5' CAACTGAYCTMYACTGTTATGA [SEQ ID NO:11]
5' CAACTGAYCTMYACTGTTATGAGCAATT [SEQ ID NO:12]

The invention still further provides a primer cocktail/probe mixture comprising the first primer cocktail according to the invention and a mixture of molecular beacon probe oligonucleotides having the following structures:

5' X_2-CCGAGCACTGAYCTMYACTGTTATGAGCTCGG-X_3 [SEQ ID NO:13]
5' X_2-CCAAGCACTGAYCTMYACTGTTATGAGCTTGG-X_3 [SEQ ID NO:14]
5' X_2-CCAAGCCAACTGAYCTMYACTGTTATGAGCAGCTTGG-X_3 [SEQ ID NO:15]

wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

The invention also provides a second primer cocktail which is suitable for use in detecting the presence of human papilloma virus type 52, type 58,

type 67 or a combination thereof in a test sample, the primer cocktail comprising the following combination of oligonucleotide molecules:

5' TGGACAGGRCGSTGTKCA [SEQ ID NO:18]
5' TGTKCAGWGTGTTGGAGA [SEQ ID NO:19]
5' X_1-TCCTCRTCTGAGCTGTCA [SEQ ID NO:16]
5' X_1-TGCTTGTCCATCTGGCCGGT [SEQ ID NO:17]
wherein X_1 represents a nucleotide sequence comprising a promoter,

In a preferred embodiment the second primer cocktail may comprise the following combination of oligonucleotide molecules:

Primer name	Sequence
HPV BP2-1	5'AATTCTAATACGACTCACTATAGGGAGAAGGT CCTCRTCTGAGCTGTCA [SEQ ID NO:48]
HPV BP2-2	5'GATGCAAGGTCGCATATGAGTGGACAGGRCGS TGTKCA [SEQ ID NO:50]
HPV BP2A-1	5'AATTCTAATACGACTCACTATAGGGAGAAGGT GCTTGTCCATCTGGCCGGT [SEQ ID NO:49]
HPV BP2A-2	5'GATGCAAGGTCGCATATGAGTGTKCAGWGTGT TGGAGA [SEQ ID NO:51]

In a further embodiment the invention also provides a primer cocktail/probe mixture comprising a second primer cocktail according to the invention and a mixture of probe oligonucleotide molecules having the following sequences:

5' CAACTGACCTAYWCTGCTATGA [SEQ ID NO:20]
5' GAAMCAACTGACCTAYWCTGCTAT [SEQ ID NO:21]

The invention further provides a primer cocktail/probe mixture comprising a second primer cocktail according to the invention and a mixture of

molecular beacon probe oligonucleotides having the following structures:

5' X_2-CCAAGCAANCAACTGACCTAYWCTGCTAGCTTGG-X_3 [SEQ ID NO:22]

5' X_2-CCAAGCACTGACCTAYWCTGCTATGAGCTTGG-X_3 [SEQ ID NO:23]

wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

The invention also provides a third primer cocktail for use in detecting the presence of human papilloma virus type 33, type 58 or a combination thereof in a test sample, the primer cocktail comprising the following combination of oligonucleotides:

5' CAACTGACCTATWCTGCTA [SEQ ID NO:26]

5' ATCCTGAACCAACTGACCTA [SEQ ID NO:27]

5' X_1-CAAGTGTRACAACARGTTA [SEQ ID NO:24]

5' X_1-GCACAGSTAGGGCACACAA [SEQ ID NO:25]

wherein X_1 represents a nucleotide sequence comprising a promoter,

In a preferred embodiment the third primer cocktail may comprise the following combination of oligonucleotide molecules:

Primer name	Sequence
HPV BP1-1	5'AATTCTAATACGACTCACTATAGGGAGAAGGC AAGTGTRACAACARGTTA [SEQ ID NO:52]
HPV BP1-2	5'GATGCAAGGTCGCATATGAGCAACTGACCTAT WCTGCTA [SEQ ID NO:54]
HPV BP1A-1	5'AATTCTAATACGACTCACTATAGGGAGAAGGG CACAGSTAGGGCACACAA [SEQ ID NO:53]

HPV BP1A-2 5'GATGCAAGGTCGCATATGAGATCCTGAACCAA CTGACCTA [SEQ ID NO:55]

In a further embodiment the invention also provides a primer cocktail/probe mixture comprising a third primer cocktail according to the invention and a mixture of probe oligonucleotide molecules having the following sequences:

5' GTGACAGCTCAGAYGAGGATGA [SEQ ID NO:28]
5' GGCCAGATGGACAAGCACAAC [SEQ ID NO:29]

The invention also provides a primer cocktail/probe mixture comprising a third primer cocktail according to the invention and a mixture of molecular beacon probe oligonucleotide molecules having the following structures:

5' X_2-CCAAGCGCCAGATGGACAAGCACAAGCTTGG-X_3 [SEQ ID NO:30]
5' X_2-CCGAGCGCCAGATGGACAAGCACAAGCTCGG-X_3 [SEQ ID NO:31]
wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

The invention still further provides a fourth primer cocktail for use in detecting the presence of human papilloma virus type 18, type 45 or a combination thereof in a test sample, the primer cocktail comprising the following combination of oligonucleotides:

5' ACGAGCAATTAAGCGAST [SEQ ID NO:35]
5' CCGACGAGCCGAACCACA [SEQ ID NO:36]
5' X_1-AGCTCAATTCTGSCKTCA [SEQ ID NO:32]
5' X_1-ACGGACACACAAAGGACA [SEQ ID NO:33]
5' X_1-GCACACCACGGACACACA [SEQ ID NO:34]

wherein X_1 represents a nucleotide sequence comprising a promoter,

In a preferred embodiment the fourth primer cocktail may comprise the following combination of oligonucleotide molecules:

Primer name	Sequence
HPVCP1-1	5'AATTCTAATACGACTCACTATAGGGAGAAGGA GCTCAATTCTGSCKTCA [SEQ ID NO:56]
HPVCP1-2	5'GATGCAAGGTCGCATATGAGACGAGCAATTAA GCGAST [SEQ ID NO:59]
HPVCP2-1	5'AATTCTAATACGACTCACTATAGGGAGAAGGA CGGACACACAAAGGACA [SEQ ID NO:57]
HPVCP2-2	5'GATGCAAGGTCGCATATGAGACGAGCAATTAA GCGAST [SEQ ID NO:60]
HPVCP3-1	5'AATTCTAATACGACTCACTATAGGGAGAAGGG CACACCACGGACACACA [SEQ ID NO:58]
HPVCP3-2	5'GATGCAAGGTCGCATATGAGCCGACGAGCCGA ACCACA [SEQ ID NO:61]

In a further embodiment the invention provides a primer cocktail/probe mixture comprising a fourth primer cocktail according to the invention and a mixture of probe oligonucleotide molecules having the following sequences:

5' AGCCCGACGAGCCGAACCACA [SEQ ID NO:37]

5' GTTGTAAGTGTGAMGSCAGAATT [SEQ ID NO:38]

The invention also provides a primer cocktail/probe mixture comprising a fourth primer cocktail according to the invention and a molecular beacon probe oligonucleotide molecule having the following structure:

5' X_2-CCAAGCAGCCCGACGAGCCGAACCACAGCTTGG-X_3 [SEQ ID

NO:39]

wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

The primer cocktails provided by the invention are mixtures of degenerate primer 1 and primer 2 oligonucleotides which collectively have specificity for two or more defined HPV types. The primer cocktails may be used to amplify HPV mRNA in a test sample by nulceic acid sequence-based amplification under standard NASBA conditions which are well known in the art. The primer cocktail/probe mixtures additionally contain degenerate probe oligonucleotides having specificity for the same HPV types. The mixtures of primer cocktails and molecular beacon probes may be used to detect HPV mRNA by real-time NASBA.

The primer cocktails and primer cocktail/probe mixtures may be supplied in the form of a solution in an appropriate buffer or they may be supplied in lyophilised or dried form and then reconstituted prior to use by the addition of a suitable buffer. Advantageously, they may be included in kits of reagents for HPV detection, as described below.

The primer cocktails of the invention allow simultaneous detection of combinations of two or more HPV types in a single NASBA reaction. The inventors' proposal of different cocktails of degenerate primer-sets that may be used to amplify transcripts from the E6 and E7 oncogenes, and in particular the ability to simultaneously detect several HPV types, represents a novel approach to the problem of detecting HPV transcripts that provides certain advantages over the prior art methods of HPV

detection, both in terms sensitivity and specificity and automation of large scale screening/detection programs.

A novel aspect of the inventors' approach is the simultaneous detection of combinations of HPV types. The combination is possible due to the similarity of the different HPV types. It is possible to construct NASBA primers against all the different oncogene types of HPV. However, a system that includes 13 different marker units is very difficult to automate on a large scale. With the use of the degenerate primer cocktails provided by the invention it is possible to screen for HPV types 16, 31, 35, 52, 58, 67, 33, 18 and 45 simultaneously using only 4 different marker units. This system is much more useful for robotic based diagnostics on a large number of patient samples.

In addition to the primer cocktails described above the invention further provides individual primer sets, i.e. pairs of primer 1 and primer 2 oligonucleotides, and also primer set/probe mixtures all of which may be used for the detection of HPV by NASBA.

Therefore, in a further aspect the invention provides three different oligonucleotide primer sets for use in the detection of HPV type 16, type 31, type 35 or a combination thereof in a test sample, as follows:

Primer set 1 comprising a first oligonucleotide primer comprising the sequence 5' X_1-AGRTCAGTTGYCTCDGGT [SEQ ID NO:1], wherein X_1 represents a nucleotide sequence comprising a promoter, and a second oliogonucleotide primer comprising the sequence 5' CCRYTGTGTCCWGWWGAA [SEQ ID NO:4].

Primer set 2 comprising a first oligonucleotide primer comprising the sequence 5' X_1-TCYGGTTYTGCTTGTCCA [SEQ ID NO:2], wherein X_1 represents a nucleotide sequence comprising a promoter, and a second oliogonucleotide primer comprising the sequence 5' TGCRTGGAGAWAYAMCTA [SEQ ID NO:5].

Primer set 3 comprising a first oligonucleotide primer comprising the sequence 5' X_1-TGTGTGCTYTGTACRCACAR [SEQ ID NO:3], wherein X_1 represents a nucleotide sequence comprising a promoter, and a second oliogonucleotide primer comprising the sequence 5' TATRTKTTAGATTTGSAACC [SEQ ID NO:6].

In each case X_1 preferably represents a nucleotide sequence comprising a T7 promoter, and even more preferably represents the nucleotide sequence AATTCTAATACGACTCACTATAGGGAGAAGG [SEQ ID NO:62].

The invention still further provides two different probe/primer mixtures for use in the detection of HPV type 16, type 31, type 35 or a combination thereof in a test sample, as follows:

Probe primer mixture 1 comprising oligonucleotide primer set 1 as defined above and at least one oligonucleotide probe comprising a nucleotide sequence selected from the group consisting of:

5' TGTAAWCATGCRTGGAGAWA [SEQ ID NO:7]
5' GAAACCSARSTGTAAWCATG [SEQ ID NO:8]
5' CATGCRTGGAGAWAYAMCTA [SEQ ID NO:9]

Probe/primer mixture 2 comprising oligonucleotide primer set 2 or 3, as defined above and at least one oligonucleotide probe selected from the group consisting of:

oligonucleotide probes comprising one of the following nucleotide sequences:

5' GACAGCTCAGAKGAGGAGGA [SEQ ID NO:10]
5' CAACTGAYCTMYACTGTTATGA [SEQ ID NO:11]
5' CAACTGAYCTMYACTGTTATGAGCAATT [SEQ ID NO:12]

and oligonucleotide probes having one of the following structures:

5' X_2-CCGAGCACTGAYCTMYACTGTTATGAGCTCGG-X_3 [SEQ ID NO:13]
5' X_2-CCAAGCACTGAYCTMYACTGTTATGAGCTTGG-X_3 [SEQ ID NO:14]
5' X_2-CCAAGCCAACTGAYCTMYACTGTTATGAGCAGCTTGG-X_3 [SEQ ID NO:15]
wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

In a still further aspect the invention provides two different oligonucleotide primer sets for use in the detection of HPV type 52, type 58 or type 67 or a combination thereof in a test sample, as follows:

Primer set 4 comprising a first oligonucleotide primer comprising the sequence 5' X_1-TCCTCRTCTGAGCTGTCA [SEQ ID NO:16], wherein X_1 represents a nucleotide sequence comprising a promoter, and a second oliogonucleotide primer comprising the sequence 5' TGGACAGGRCGSTGTKCA [SEQ ID NO:18].

Primer set 5 comprising a first oligonucleotide primer comprising the sequence 5' X_1-TGCTTGTCCATCTGGCCGGT [SEQ ID NO:17], wherein X_1 represents a nucleotide sequence comprising a promoter, and a second

oliogonucleotide primer comprising the sequence 5' TGTKCAGWGTGTTGGAGA [SEQ ID NO:19].

In each case X_1 preferably represents a nucleotide sequence comprising a T7 promoter, and even more preferably represents the nucleotide sequence AATTCTAATACGACTCACTATAGGGAGAAGG [SEQ ID NO:62].

The invention further provides a probe/primer mixture for use in the detection of HPV type 52, type 58, type 67 or a combination thereof in a test sample, as follows:

Probe/primer mixture 3 comprising oligonucleotide primer set 4 or 5, as defined above, and at least one oligonucleotide probe selected from the group consisting of:

oligonucleotide probes comprising one of the following nucleotide sequences:

5' CAACTGACCTAYWCTGCTATGA [SEQ ID NO:20]
5' GAAMCAACTGACCTAYWCTGCTAT [SEQ ID NO:21]

and oligonucleotide probes having one of the following structures:

5' X_2-CCAAGCAANCAACTGACCTAYWCTGCTAGCTTGG-X_3 [SEQ ID NO:22]
5' X_2-CCAAGCACTGACCTAYWCTGCTATGAGCTTGG-X_3 [SEQ ID NO:23]
wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

In a still further aspect the invention provides

two oligonucleotide primer sets for use in the detection of HPV type 33, type 58 or a combination thereof in a test sample, as follows:

Primer set 6 comprising a first oligonucleotide primer comprising the sequence 5' X_1-CAAGTGTRACAACARGTTA [SEQ ID NO:24], wherein X_1 represents a nucleotide sequence comprising a promoter, and a second oliogonucleotide primer comprising the sequence 5' CAACTGACCTATWCTGCTA [SEQ ID NO:26].

Primer set 7 comprising a first oligonucleotide primer comprising the sequence 5' X_1-GCACAGSTAGGGCACACAA [SEQ ID NO:25], wherein X_1 represents a nucleotide sequence comprising a promoter, and a second oliogonucleotide primer comprising the sequence 5'ATCCTGAACCAACTGACCTA [SEQ ID NO:27].

In each case X_1 preferably represents a nucleotide sequence comprising a T7 promoter, and even more preferably represents the nucleotide sequence AATTCTAATACGACTCACTATAGGGAGAAGG [SEQ ID NO:62].

The invention further provides a probe/primer mixture for use in the detection of HPV type 33, type 58 or a combination thereof in a test sample, as follows:

Probe/primer mixture 4 comprising oligonucleotide primer set 6 or 7, as defined above, and at least one oligonucleotide probe selected from the group consisting of:

oligonucleotide probes comprising one of the following nucleotide sequences:

5' GTGACAGCTCAGAYGAGGATGA [SEQ ID NO:28]

5' GGCCAGATGGACAAGCACAAC [SEQ ID NO:29]

and oligonucleotide probes having one of the following structures:

5' X_2-CCAAGCGCCAGATGGACAAGCACAAGCTTGG-X_3 [SEQ ID NO:30]

5' X_2-CCGAGCGCCAGATGGACAAGCACAAGCTCGG-X_3 [SEQ ID NO:31]

wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

In a still further aspect the invention provides three different oligonucleotide primer sets for use in the detection of HPV type 45, type 18 or a combination thereof in a test sample, as follows:

Primer set 8 comprising a first oligonucleotide primer comprising the sequence 5' X_1-AGCTCAATTCTGSCKTCA [SEQ ID NO:32], wherein X_1 represents a nucleotide sequence comprising a promoter, and a second oliogonucleotide primer comprising the sequence 5' ACGAGCAATTAAGCGAST [SEQ ID NO:35].

Primer set 9 comprising a first oligonucleotide primer comprising the sequence 5' X_1-ACGGACACACAAAGGACA [SEQ ID NO:33], wherein X_1 represents a nucleotide sequence comprising a promoter, and a second oliogonucleotide primer comprising the sequence 5' ACGAGCAATTAAGCGAST [SEQ ID NO:35].

Primer set 10 comprising a first oligonucleotide primer comprising the sequence 5' X_1-GCACACCACGGACACACA [SEQ ID NO:34], wherein X_1 represents a nucleotide sequence comprising a

promoter, and a second oliogonucleotide primer comprising the sequence 5' CCGACGAGCCGAACCACA [SEQ ID NO:36].

In each case X_1 preferably represents a nucleotide sequence comprising a T7 promoter, and even more preferably represents the nucleotide sequence AATTCTAATACGACTCACTATAGGGAGAAGG [SEQ ID NO:62].

The invention still further provides two different probe/primer mixtures for use in the detection of HPV type 45, type 18 or a combination thereof in a test sample, as follows:

Probe/primer mixture 5 comprising oligonucleotide primer set 8, as defined above, and at least one of: an oligonucleotide probe comprising the nucleotide sequence 5' AGCCCGACGAGCCGAACCACA [SEQ ID NO:37] or an oligonucleotide probe having the structure 5' X_2-CCAAGCAGCCCGACGAGCCGAACCACAGCTTGG-X_3 [SEQ ID NO:39] wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

Probe/primer mixture 6 comprising oligonucleotide primer set 9 or 10, as defined above, and at least one of: an oligonucleotide probe comprising the nucleotide sequence 5' GTTGTAAGTGTGAMGSCAGAATT [SEQ ID NO:38] an oligonucleotide probe having the structure 5' X_2-CCAAGCAGCCCGACGAGCCGAACCACAGCTTGG-X_3 [SEQ ID NO: 39] wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

The primer sets and probe/primer mixtures may also be supplied in the form of a solution in an appropriate buffer or in a lyophilised or dried form which may be reconstituted prior to use by the addition of a suitable buffer. Advantageously, they may be included in kits of reagents for HPV detection, as described below.

Also provided by the invention are reagent kits for use in the detection of HPV by NASBA, the kits comprising one or more oligonucleotide primer cocktails or primer sets acccording to the invention. The reagent kits may further comprise a mixture of enzymes required for the NASBA reaction, specifically an enzyme mixture containing an RNA directed DNA polymerase (e.g. a reverse transcriptase), a ribonuclease that hydrolyses the RNA strand of an RNA-DNA hybrid without hydrolysing single or double stranded RNA or DNA (e.g. RNaseH) and an RNA polymerase. The RNA polymerase should be one which recognises the promoter sequence present in the 5' terminal region of the primer 1 oligonucleotides in the oligonucleotide primer cocktails or primer sets supplied in the reagent kit. The kit may also comprise a supply of NASBA buffer containing the ribonucleosides and deoxyribonucleosides required for RNA and DNA synthesis. Details of the composition of a standard NASBA reaction buffer are given in the accompanying examples.

In certain embodiments the kit may further contain one or more capture probes, comprising a probe oligonucleotide attached to a solid support as described above, for immobilising the products of a specific NASBA reaction. The kit may still further contain labelled generic detection probes. Advantageously, the detection probes may comprise a sequence of nucleotides complementary to a non-HPV

sequence present at the 5' terminal end of the primer 2 oligonucleotides present in the reagent kit.

In still further embodiments the kit may further contain one or more molecular beacon probes according to the invention. The molecular beacon probes may be supplied as a seperate reagent within the kit or may be supplied in a primer cocktail/probe or primer set/probe mixture.

In a still further aspect the invention provides methods for the detection of HPV in test samples using the NASBA technique. These methods generally comprise the following steps:

(a) providing a reaction medium comprising a primer cocktail or a primer set according to the invention, an RNA directed DNA polymerase, a ribonuclease that hydrolyses the RNA strand of an RNA-DNA hybrid without hydrolysing single or double stranded RNA or DNA, an RNA polymerase that recognises said promoter, and ribonucleoside and deoxyribonucleoside triphosphates;

(b) incubating said reaction medium with a preparation of nucleic acid isolated from a test sample suspected of containing HPV under reaction conditions which permit a NASBA amplification reaction; and

(c) detecting and/or quantitatively measuring any HPV-specific product of the NASBA amplification reaction.

The 'test sample suspected of containing HPV' will most commonly be a clinical sample, for example a cervical scraping in the cervical screening field.

The NASBA reaction will preferably be carried out on a preparation of nucleic acid isolated from the test sample. This preparation of nucleic acid must include mRNA, however it need not be a preparation of

purified poly A+ mRNA and preparations of total RNA or even preparations of total nucleic acid containing both RNA and genomic DNA are also suitable as starting material for a NASBA reaction. Essentially any technique known in the art for the isolation of a preparation of nucleic acid including mRNA may be used to isolate nucleic acid from the test sample. A preferred technique is the "Boom" isolation method described in US-A-5,234,809 and EP-B-0389,063. This method, which can be used to isolate a nucleic acid preparation containing both RNA and DNA, is based on the nucleic acid binding properties of silicon dioxide particles in the presence of the chaotropic agent guanidine thiocyanate (GuSCN).

Detection of the specific product(s) of the NASBA reaction (i.e. sense and/or antisense copies of the target RNA) may be carried out in a number of different ways. In one approach the NASBA product(s) may be detected with the use of an HPV-specific hybridisation probe capable of specifically annealing to the NASBA product. The hybridisation probe may be attached to a revealing label, for example a fluorescent, luminescent, radioactive or chemiluminescent compound or an enzyme label or any other type of label known to those of ordinary skill in the art. The precise nature of the label is not critical, but it should be capable of producing a signal detectable by external means, either by itself or in conjuction with one or more additional substances (e.g. the substrate for an enzyme).

Also within the scope of the invention is so-called 'real-time NASBA' which allows continuous monitoring of the formation of the product of the NASBA reaction over the course of the reaction. This may be achieved using a 'molecular beacons' probe comprising an HPV-specific sequence capable of annealing to the NASBA product, a hairpin forming

oligonucleotide sequence and a pair of fluorescer/quencher moieties, as described in WO 95/13399. If the molecular beacons probe is added to the reaction mixture prior to amplification it may be possible to monitor the formation of the NASBA product in real-time (see Leone *et al.*, Nucleic Acids Research., 1998, vol: 26, pp 2150-2155).

In a further approach, the molecular beacons technology may be incorporated into the primer 2 oligonucleotide allowing real-time monitoring of the NASBA reaction without the need for a separate hybridisation probe.

In a still further approach the products of the NASBA reaction may be monitored using a generic labelled detection probe which hybridises to a nucleotide sequence in the 5' terminus of primer 2. This is equivalent to the 'NucliSens™' detection system supplied by Organon Teknika. In this system specificity for NASBA products derived from the target HPV mRNA may be conferred by using HPV-specific capture probes comprising probe oligonucleotides as described herein attached to a solid support such as a magnetic microbead. Most preferably the generic labelled detection probe is the ECL™ detection probe supplied by Organon Teknika. NASBA amplicons are hybridized to the HPV-specific capture probes and the generic ECL probe (via a complementary sequence on primer 2). Following hybridization the bead/amplicon/ECL probe complexes may be captured at the magnet electrode of an automatic ECL reader (e.g. the NucliSens™ reader supplied by Organon Teknika. Subsequently, a voltage pulse triggers the ECL™ reaction.

The invention provides a number of HPV-specific oligonucleotide probes, including molecular beacons probes, an it will be readily apparent to one of ordinary skill in the art from the foregoing

description which probes or mixtures of probes are suitable for use with a given primer cocktail or primer set.

The present invention will be further understood with reference to the following Examples:

Example 1-Design of NASBA primer cocktails

The degenerated primer sets were constructed as follows:

We used the newly released program (year 2000) included in the series of Blast programs to compare all the different E6 and E7 genes from the 13 HPV types: HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68. From the raw data of the compared sequences we carefully performed a manual search for areas that fulfilled the following criteria in addition to the similarity:

1. Reduced hairpin formation (less than 3 bases)
2. Reduced dimer formation (less than 3 bases)
3. Ideal internal stability
4. Primer length (without the attached T7 RNA polymerase promotor sequence) between 20 and 25.
5. Probe length between 20 and 25.
6. Length of the amplification product between 120 and 250.
7. Must have 1 or 2 A bases as the final nucleotide at the 3'-end
8. Must have G+C content of 40-60%
9. The first 10 nts following the promotor sequence of primer 1 must be purine rich.
10. No stretches of pyrimidines in the first 10-12 nucleotides.
11. No false priming either in the sense or in the

antisense of the targeted sequence.

12. The stability of the primer-set compared to the product has to be optimal.
13. The delta G value of the two different primer-set has to be equal.
14. The Tm difference has to be under 4 to 5°C.
15. The annealing temperature must be below 55°C.
16. The number of mixed bases sites have to be under 4.

Example 2-Real-time NASBA

The final real-time NASBA reaction buffer consists of the following components:

NASBA buffer: 3 ng/nl molecular beacon probe, 1 mM of each dNTP, 2 mM of ATP, UTP and CTP, 1.5 mM GTP, and 0.5 mM ITP, 0.5 mM dithiotreitol, 70 mM KCl, 12 mM $MgCl_2$, 40 mM Tric-HCl (pH 8.5).

The primer mix solution consist of 45% DMSO and 0.2 µM each of the antisense and sense primers.

The enzyme mix consists of 375 mM sorbitol, 2.5 µg BSA, 0.08 U RNase H, 32 U T7 RNA polymerase and 6.4 U AMV-reverse transcriptase.

Claims:

1. An oligonucleotide molecule for use in determining the presence of human papilloma virus type 16, type 31, type 35 or a combination thereof in a test sample, said oligonucleotide being selected from any of groups (i) to (iv) consisting of:

(i) oligonucleotides comprising one of the following nucleotide sequences:

5' X_1-AGRTCAGTTGYCTCDGGT [SEQ ID NO:1]
5' X_1-TCYGGTTYTGCTTGTCCA [SEQ ID NO:2]
5' X_1-TGTGTGCTYTGTACRCACAR [SEQ ID NO:3]

wherein X_1 represents a nucleotide sequence comprising a promoter

(ii) oligonucleotides comprising one of the following nucleotide sequences:

5' CCRYTGTGTCCWGWWGAA [SEQ ID NO:4]
5' TGCRTGGAGAWAYAMCTA [SEQ ID NO:5]
5' TATRTKTTAGATTTGSAACC [SEQ ID NO:6]

(iii)oligonucleotides comprising one of the following nucleotide sequences:

5' TGTAAWCATGCRTGGAGAWA [SEQ ID NO:7]
5' GAAACCSARSTGTAAWCATG [SEQ ID NO:8]
5' CATGCRTGGAGAWAYAMCTA [SEQ ID NO:9]
5' GACAGCTCAGAKGAGGAGGA [SEQ ID NO:10]
5' CAACTGAYCTMYACTGTTATGA [SEQ ID NO:11]
5' CAACTGAYCTMYACTGTTATGAGCAATT [SEQ ID NO:12]

(iv) oligonucleotides comprising one of the following structures:

5' X_2-CCGAGCACTGAYCTMYACTGTTATGAGCTCGG-X_3 [SEQ ID NO:13]
5' X_2-CCAAGCACTGAYCTMYACTGTTATGAGCTTGG-X_3 [SEQ ID NO:14]
5' X_2-CCAAGCCAACTGAYCTMYACTGTTATGAGCAGCTTGG-X_3 [SEQ ID NO:15]

wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

2. An oligonucleotide molecule according to claim 1 wherein X_1 represents a nucleotide sequence comprising a T7 promoter.

3. An oligonucleotide molecule according to claim 1 which comprises a sequence selected from the group consisting of:

5' AATTCTAATACGACTCACTATAGGGAGAAGGAGRTCAGTTGYCTCDGGT [SEQ ID NO:42]
5' GATGCAAGGTCGCATATGAGCCRYTGTGTCCWGWWGAA [SEQ ID NO:45]
5' AATTCTAATACGACTCACTATAGGGAGAAGGTCYGGTTYTGCTTGTCCA [SEQ ID NO:43]
5' GATGCAAGGTCGCATATGAGTGCRTGGAGAWAYAMCTA [SEQ ID NO:46]
5' AATTCTAATACGACTCACTATAGGGAGAAGGTGTGTGCTYTGTACRCACAR [SEQ ID NO:44]
5' GATGCAAGGTCGCATATGAGTATRTKTTAGATTTGSAACC [SEQ ID NO:47]

4. An oligonucleotide molecule for use in determining the presence of human papilloma virus type 52, type 58, type 67 or a combination thereof in a test sample, said oligonucleotide being selected from

any of groups (i) to (iv) consisting of:

(i) oligonucleotides comprising one of the following nucleotide sequences:

5' X_1-TCCTCRTCTGAGCTGTCA [SEQ ID NO:16]
5' X_1-TGCTTGTCCATCTGGCCGGT [SEQ ID NO:17]
wherein X_1 represents a nucleotide sequence comprising a promoter,

(ii) oligonucleotides comprising one of the following nucleotide sequences:

5' TGGACAGGRCGSTGTKCA [SEQ ID NO:18]
5' TGTKCAGWGTGTTGGAGA [SEQ ID NO:19]

(iii) oligonucleotides comprising one of the following nucleotide sequences:

5' CAACTGACCTAYWCTGCTATGA [SEQ ID NO:20]
5' GAAMCAACTGACCTAYWCTGCTAT [SEQ ID NO:21]

(iv) oligonucleotides comprising one of the following structures:

5' X_2-CCAAGCAANCAACTGACCTAYWCTGCTAGCTTGG-X_3 [SEQ ID NO:22]
5' X_2-CCAAGCACTGACCTAYWCTGCTATGAGCTTGG-X_3 [SEQ ID NO:23]
wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

5. An oligonucleotide molecule according to claim 4 wherein X_1 represents a nucleotide sequence comprising a T7 promoter.

6. An oligonucleotide molecule according to claim 4 which comprises a sequence selected from the group consisting of:

5' AATTCTAATACGACTCACTATAGGGAGAAGGTCCTCRTCTGAGCTGTCA [SEQ ID NO:48]

5' GATGCAAGGTCGCATATGAGTGGACAGGRCGSTGTKCA [SEQ ID NO:50]

5' AATTCTAATACGACTCACTATAGGGAGAAGGTGCTTGTCCATCTGGCCGGT [SEQ ID NO:49]

5' GATGCAAGGTCGCATATGAGTGTKCAGWGTGTTGGAGA [SEQ ID NO:50]

7. An oligonucleotide molecule for use in determining the presence of human papilloma virus type 33, type 58 or a combination thereof in a test sample, said oligonucleotide being selected from any of groups (i) to (iv) consisting of:

(i) oligonucleotides comprising one of the following nucleotide sequences:

5' X_1-CAAGTGTRACAACARGTTA [SEQ ID NO:24]

5' X_1-GCACAGSTAGGGCACACAA [SEQ ID NO:25]

wherein X_1 represents a nucleotide sequence comprising a promoter,

(ii) oligonucleotides comprising one of the following nucleotide sequences:

5' CAACTGACCTATWCTGCTA [SEQ ID NO:26]

5' ATCCTGAACCAACTGACCTA [SEQ ID NO:27]

(iii) oligonucleotides comprising one of the following nucleotide sequences:

5'GTGACAGCTCAGAYGAGGATGA [SEQ ID NO:28]

5'GGCCAGATGGACAAGCACAAC [SEQ ID NO:29]

(iv) oligonucleotides comprising one of the following structures:

5' X_2-CCAAGCGCCAGATGGACAAGCACAAGCTTGG-X_3 [SEQ ID NO:30]
5' X_2-CCGAGCGCCAGATGGACAAGCACAAGCTCGG-X_3 [SEQ ID NO:31]
wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

8. An oligonucleotide molecule according to claim 7 wherein X_1 represents a nucleotide sequence comprising a T7 promoter.

9. An oligonucleotide molecule according to claim 7 which comprises a sequence selected from the group consisting of:

5' AATTCTAATACGACTCACTATAGGGAGAAGGCAAGTGTRACAACARGTTA [SEQ ID NO:52]
5' GATGCAAGGTCGCATATGAGCAACTGACCTATWCTGCTA [SEQ ID NO:54]
5' AATTCTAATACGACTCACTATAGGGAGAAGGGCACAGSTAGGGCACACAA [SEQ ID NO:53]
5' GATGCAAGGTCGCATATGAGATCCTGAACCAACTGACCTA [SEQ ID NO:55]

10. An oligonucleotide molecule for use in determining the presence of human papilloma virus type 18, type 45 or a combination thereof in a test sample, said oligonucleotide being selected from any of groups (i) to (iv) consisting of:

(i) oligonucleotides comprising one of the following

nucleotide sequences:

5' X_1-AGCTCAATTCTGSCKTCA [SEQ ID NO:32]
5' X_1-ACGGACACACAAAGGACA [SEQ ID NO:33]
5' X_1-GCACACCACGGACACACA [SEQ ID NO:34]
wherein X_1 represents a nucleotide sequence comprising a promoter,

(ii) oligonucleotides comprising one of the following nucleotide sequences:

5' ACGAGCAATTAAGCGAST [SEQ ID NO:35]
5' CCGACGAGCCGAACCACA [SEQ ID NO:36]

(iii) oligonucleotides comprising one of the following nucleotide sequences:

5'AGCCCGACGAGCCGAACCACA [SEQ ID NO:37]
5'GTTGTAAGTGTGAMGSCAGAATT [SEQ ID NO:38]

(iv) oligonucleotides having the following structure:

5' X_2-CCAAGCAGCCCGACGAGCCGAACCACAGCTTGG-X_3 [SEQ ID NO:39]
wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

11. An oligonucleotide molecule according to claim 10 wherein X_1 represents a nucleotide sequence comprising a T7 promoter.

12. An oligonucleotide molecule according to claim 10 which comprises a sequence selected from the group consisting of:

5' AATTCTAATACGACTCACTATAGGGAGAAGGAGCTCAATTCTGSCKTCA [SEQ ID NO:56]

5' GATGCAAGGTCGCATATGAGACGAGCAATTAAGCGAST [SEQ ID NO:59]

5' AATTCTAATACGACTCACTATAGGGAGAAGGACGGACACACAAAGGACA [SEQ ID NO:57]

5' GATGCAAGGTCGCATATGAGACGAGCAATTAAGCGAST [SEQ ID NO:60]

5' AATTCTAATACGACTCACTATAGGGAGAAGGGCACACCACGGACACACA [SEQ ID NO:58]

5' GATGCAAGGTCGCATATGAGCCGACGAGCCGAACCACA [SEQ ID NO:61]

13. An oligonucleotide primer cocktail for use in the detection of human papilloma virus type 16, type 31, type 35 or a combination thereof in a test sample, the primer cocktail comprising a combination of oligonucleotide molecules having the following sequences:

5' CCRYTGTGTCCWGWWGAA [SEQ ID NO:4]

5' TGCRTGGAGAWAYAMCTA [SEQ ID NO:5]

5' TATRTKTTAGATTTGSAACC [SEQ ID NO:6]

5' X_1-AGRTCAGTTGYCTCDGGT [SEQ ID NO:1]

5' X_1-TCYGGTTYTGCTTGTCCA [SEQ ID NO:2]

5' X_1-TGTGTGCTYTGTACRCACAR [SEQ ID NO:3]

wherein X_1 represents a nucleotide sequence comprising a promoter,

14. An oligonucleotide primer cocktail as claimed in claim 13 wherein X_1 represents a nucleotide sequence comprising a T7 promoter.

15. An oligonucleotide primer cocktail as claimed in claim 14 wherein X_1 represents a nucleotide sequence comprising the following sequence:
5'AATTCTAATACGACTCACTATAGGGAGAAGG [SEQ ID NO:62]

16. An oligonucleotide primer cocktail as claimed in claim 13 which comprises the following combination of oligonucleotide molecules:

5' AATTCTAATACGACTCACTATAGGGAGAAGGAGRTCAGTTGYCTCDGGT [SEQ ID NO:42]
5' GATGCAAGGTCGCATATGAGCCRYTGTGTCCWGWWGAA [SEQ ID NO:45]
5' AATTCTAATACGACTCACTATAGGGAGAAGGTCYGGTTYTGCTTGTCCA [SEQ ID NO:43]
5' GATGCAAGGTCGCATATGAGTGCRTGGAGAWAYAMCTA [SEQ ID NO:46]
5' AATTCTAATACGACTCACTATAGGGAGAAGGTGTGTGCTYTGTACRCACAR [SEQ ID NO:44]
5' GATGCAAGGTCGCATATGAGTATRTKTTAGATTTGSAACC [SEQ ID NO:47]

17. A primer cocktail/probe mixture for use in the detection of human papilloma virus type 16, type 31, type 35 or a combination thereof in a test sample comprising an oligonucleotide primer cocktail as claimed in any one of claims 13 to 16 and oligonucleotide probe molecules having the following sequences:

5' TGTAAWCATGCRTGGAGAWA [SEQ ID NO:7]
5' GAAACCSARSTGTAAWCATG [SEQ ID NO:8]
5' CATGCRTGGAGAWAYAMCTA [SEQ ID NO:9]
5' GACAGCTCAGAKGAGGAGGA [SEQ ID NO:10]
5' CAACTGAYCTMYACTGTTATGA [SEQ ID NO:11]
5' CAACTGAYCTMYACTGTTATGAGCAATT [SEQ ID NO:12]

18. A primer cocktail/probe mixture for use in the detection of human papilloma virus type 16, type 31, type 35 or a combination thereof in a test sample comprising an oligonucleotide primer cocktail as comprising an oligonucleotide primer cocktail as

claimed in any one of claims 13 to 16 and oligonucleotide probe molecules having the following structures:

5' X_2-CCGAGCACTGAYCTMYACTGTTATGAGCTCGG-X_3 [SEQ ID NO:13]

5' X_2-CCAAGCACTGAYCTMYACTGTTATGAGCTTGG-X_3 [SEQ ID NO:14]

5' X_2-CCAAGCCAACTGAYCTMYACTGTTATGAGCAGCTTGG-X_3 [SEQ ID NO:15]

wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

19. An oligonucleotide primer cocktail for use in detecting the presence of human papilloma virus type 52, type 58, type 67 or a combination thereof in a test sample, the primer cocktail comprising a combination of oligonucleotide molecules having the following sequences:

5' TGGACAGGRCGSTGTKCA [SEQ ID NO:18]

5' TGTKCAGWGTGTTGGAGA [SEQ ID NO:19]

5' X_1-TCCTCRTCTGAGCTGTCA [SEQ ID NO:16]

5' X_1-TGCTTGTCCATCTGGCCGGT [SEQ ID NO:17]

wherein X_1 represents a nucleotide sequence comprising a promoter,

20. An oligonucleotide primer cocktail as claimed in claim 19 wherein X_1 represents a nucleotide sequence comprising a T7 promoter.

21. An oligonucleotide primer cocktail as claimed in claim 19 wherein X_1 represents a nucleotide sequence comprising the following sequence:

5'AATTCTAATACGACTCACTATAGGGAGAAGG [SEQ ID NO:62]

22. An oligonucleotide primer cocktail as claimed in claim 19 which comprises the following combination of oligonucleotide molecules:

5' AATTCTAATACGACTCACTATAGGGAGAAGGTCCTCRTCTGAGCTGTCA [SEQ ID NO:48]
5' GATGCAAGGTCGCATATGAGTGGACAGGRCGSTGTKCA [SEQ ID NO:50]
5' AATTCTAATACGACTCACTATAGGGAGAAGGTGCTTGTCCATCTGGCCGGT [SEQ ID NO:49]
5' GATGCAAGGTCGCATATGAGTGTKCAGWGTGTTGGAGA [SEQ ID NO:51]

23. A primer cocktail/probe mixture for use in detecting the presence of human papilloma virus type 52, type 58, type 67 or a combination thereof in a test sample comprising an oligonucleotide primer cocktail as claimed in any one of claims 19 to 22 and oligonucleotide probe molecules having the following sequences:

5' CAACTGACCTAYWCTGCTATGA [SEQ ID NO:20]
5' GAAMCAACTGACCTAYWCTGCTAT [SEQ ID NO:21]

24. A primer cocktail/probe mixture for use in detecting the presence of human papilloma virus type 52, type 58, type 67 or a combination thereof in a test sample comprising an oligonucleotide primer cocktail as claimed in any one of claims 19 to 22 and oligonucleotide probe molecules having the following structures:

5' X_2-CCAAGCAANCAACTGACCTAYWCTGCTAGCTTGG-X_3 [SEQ ID NO:22]
5' X_2-CCAAGCACTGACCTAYWCTGCTATGAGCTTGG-X_3 [SEQ ID

NO:23]

wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

25. An oligonucleotide primer cocktail for use in detecting the presence of human papilloma virus type 33, type 58 or a combination thereof in a test sample, the primer cocktail comprising a combination of oligonucleotide molecules having the following sequences:

5' CAACTGACCTATWCTGCTA [SEQ ID NO:26]
5' ATCCTGAACCAACTGACCTA [SEQ ID NO:27]
5' X_1-CAAGTGTRACAACARGTTA [SEQ ID NO:24]
5' X_1-GCACAGSTAGGGCACACAA [SEQ ID NO:25]
wherein X_1 represents a nucleotide sequence comprising a promoter,

26. An oligonucleotide primer cocktail as claimed in claim 25 wherein X_1 represents a nucleotide sequence comprising a T7 promoter.

27. An oligonucleotide primer cocktail as claimed in claim 26 wherein X_1 represents a nucleotide sequence comprising the following sequence:
5'AATTCTAATACGACTCACTATAGGGAGAAGG [SEQ ID NO:62].

28. An oligonucleotide primer cocktail as claimed in claim 25 which comprises the following combination of oligonucleotide molecules:

5' AATTCTAATACGACTCACTATAGGGAGAAGGCAAGTGTRACAACARGTTA [SEQ ID NO:52]
5' GATGCAAGGTCGCATATGAGCAACTGACCTATWCTGCTA [SEQ ID

NO:54]

5' AATTCTAATACGACTCACTATAGGGAGAAGGGCACAGSTAGGGCACACAA [SEQ ID NO:53]

5' GATGCAAGGTCGCATATGAGATCCTGAACCAACTGACCTA [SEQ ID NO:55]

29. A primer cocktail/probe mixture for use in detecting the presence of human papilloma virus type 33, type 58 or a combination thereof in a test sample comprising an oligonucleotide primer cocktail as claimed in any one of claims 25 to 28 and oligonucleotide probe molecules having the following sequences:

5' GTGACAGCTCAGAYGAGGATGA [SEQ ID NO:28]

5' GGCCAGATGGACAAGCACAAC [SEQ ID NO:29]

30. A primer cocktail/probe mixture for use in detecting the presence of human papilloma virus type 33, type 58 or a combination thereof in a test sample comprising an oligonucleotide primer cocktail as claimed in any one of claims 25 to 28 and oligonucleotide probe molecules having the following structures:

5' X_2-CCAAGCGCCAGATGGACAAGCACAAGCTTGG-X_3 [SEQ ID NO:30]

5' X_2-CCGAGCGCCAGATGGACAAGCACAAGCTCGG-X_3 [SEQ ID NO:31]

wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

31. An oligonucleotide a primer cocktail for use in detecting the presence of human papilloma virus type 18, type 45 or a combination thereof in a test

sample, the primer cocktail comprising a combination of oligonucleotide molecules having the following sequences:

5' ACGAGCAATTAAGCGAST [SEQ ID NO:35]
5' CCGACGAGCCGAACCACA [SEQ ID NO:36]
5' X_1-AGCTCAATTCTGSCKTCA [SEQ ID NO:32]
5' X_1-ACGGACACACAAAGGACA [SEQ ID NO:33]
5' X_1-GCACACCACGGACACACA [SEQ ID NO:34]

wherein X_1 represents a nucleotide sequence comprising a promoter,

32. An oligonucleotide primer cocktail as claimed in claim 31 wherein X_1 represents a nucleotide sequence comprising a T7 promoter.

33. An oligonucleotide primer cocktail as claimed in claim 32 wherein X_1 represents a nucleotide sequence comprising the following sequence:
5'AATTCTAATACGACTCACTATAGGGAGAAGG [SEQ ID NO:62].

34. An oligonucleotide a primer cocktail as claimed in claim 31 which comprises the following combination of oligonucleotide molecules:

5' AATTCTAATACGACTCACTATAGGGAGAAGGAGCTCAATTCTGSCKTCA [SEQ ID NO:56]
5' GATGCAAGGTCGCATATGAGACGAGCAATTAAGCGAST [SEQ ID NO:59]
5' AATTCTAATACGACTCACTATAGGGAGAAGGACGGACACACAAAGGACA [SEQ ID NO:57]
5' GATGCAAGGTCGCATATGAGACGAGCAATTAAGCGAST [SEQ ID NO:60]
5' AATTCTAATACGACTCACTATAGGGAGAAGGGCACACCACGGACACACA [SEQ ID NO:58]
5' GATGCAAGGTCGCATATGAGCCGACGAGCCGAACCACA [SEQ ID NO:61]

35. A primer cocktail/probe mixture for use in detecting the presence of human papilloma virus type 18, type 45 or a combination thereof in a test sample comprising an oligonucleotide primer cocktail as claimed in any one of claims 31 to 34 and oligonucleotide probe molecules having the following sequences:

5' AGCCCGACGAGCCGAACCACA [SEQ ID NO:37]

5' GTTGTAAGTGTGAMGSCAGAATT [SEQ ID NO:38]

36. A primer cocktail/probe mixture for use in detecting the presence of human papilloma virus type 18, type 45 or a combination thereof in a test sample comprising an oligonucleotide primer cocktail as claimed in any one of claims 31 to 34 and an oligonucleotide probe molecules having the structure:

5' X_2-CCAAGCAGCCCGACGAGCCGAACCACAGCTTGG-X_3 [SEQ ID NO:39]

wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

37. An oligonucleotide primer set for use in the detection of HPV type 16, type 31, type 35 or a combination thereof in a test sample, comprising a first oligonucleotide primer comprising the sequence 5' X_1-AGRTCAGTTGYCTCDGGT [SEQ ID NO:1], wherein X_1 represents a nucleotide sequence comprising a promoter, and a second oliogonucleotide primer comprising the sequence 5' CCRYTGTGTCCWGWWGAA [SEQ ID NO:4].

38. An oligonucleotide primer set for use in the

detection of HPV type 16, type 31, type 35 or a combination thereof in a test sample comprising a first oligonucleotide primer comprising the sequence 5' X_1-TCYGGTTYTGCTTGTCCA [SEQ ID NO:2], wherein X_1 represents a nucleotide sequence comprising a promoter, and a second oliogonucleotide primer comprising the sequence 5' TGCRTGGAGAWAYAMCTA [SEQ ID NO:5].

39. An oligonucleotide primer set for use in the detection of HPV type 16, type 31, type 35 or a combination thereof in a test sample comprising a first oligonucleotide primer comprising the sequence 5' X_1-TGTGTGCTYTGTACRCACAR [SEQ ID NO:3], wherein X_1 represents a nucleotide sequence comprising a promoter, and a second oliogonucleotide primer comprising the sequence 5' TATRTKTTAGATTTGSAACC [SEQ ID NO:6].

40. An oligonucleotide primer set according to any one of claims 37 to 39 wherein X_1 represents a nucleotide sequence comprising a T7 promoter.

41. An oligonucleotide primer set according to claim 40 wherein X_1 represents the nucleotide sequence AATTCTAATACGACTCACTATAGGGAGAAGG [SEQ ID NO:62].

42. A probe/primer mixture for use in the detection of HPV type 16, type 31, type 35 or a combination thereof in a test sample, the mixture comprising an oligonucleotide primer set according to claim 33 and at least one oligonucleotide probe comprising a nucleotide sequence selected from the group consisting of:

5' TGTAAWCATGCRTGGAGAWA [SEQ ID NO:7]

5' GAAACCSARSTGTAAWCATG [SEQ ID NO:8]

5' CATGCRTGGAGAWAYAMCTA [SEQ ID NO:9]

43. A probe/primer mixture for use in the detection of HPV type 16, type 31, type 35 or a combination thereof in a test sample, the mixture comprising an oligonucleotide primer set according to claim 38 or claim 39 and at least one oligonucleotide probe selected from the group consisting of:

oligonucleotide probes comprising one of the following nucleotide sequences:

5' GACAGCTCAGAKGAGGAGGA [SEQ ID NO:10]
5' CAACTGAYCTMYACTGTTATGA [SEQ ID NO:11]
5' CAACTGAYCTMYACTGTTATGAGCAATT [SEQ ID NO:12]

and oligonucleotide probes having one of the following structures:

5' X_2-CCGAGCACTGAYCTMYACTGTTATGAGCTCGG-X_3 [SEQ ID NO:13]
5' X_2-CCAAGCACTGAYCTMYACTGTTATGAGCTTGG-X_3 [SEQ ID NO:14]
5' X_2-CCAAGCCAACTGAYCTMYACTGTTATGAGCAGCTTGG-X_3 [SEQ ID NO:15]

wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

44. An oligonucleotide primer set for use in the detection of HPV type 52, type 58 or type 67 or a combination thereof in a test sample comprising a first oligonucleotide primer comprising the sequence 5' X_1-TCCTCRTCTGAGCTGTCA [SEQ ID NO:16], wherein X_1 represents a nucleotide sequence comprising a

promoter, and a second oligonucleotide primer comprising the sequence 5' TGGACAGGRCGSTGTKCA [SEQ ID NO:18].

45. An oligonucleotide primer set for use in the detection of HPV type 52, type 58, type 67 or a combination thereof in a test sample comprising a first oligonucleotide primer comprising the sequence 5' X_1-TGCTTGTCCATCTGGCCGGT [SEQ ID NO:17], wherein X_1 represents a nucleotide sequence comprising a promoter, and a second oligonucleotide primer comprising the sequence 5' TGTKCAGWGTGTTGGAGA [SEQ ID NO:19].

46. An oligonucleotide primer set according to claim 44 or claim 45 wherein X_1 represents a nucleotide sequence comprising a T7 promoter.

47. An oligonucleotide primer set according to claim 46 wherein X_1 represents the nucleotide sequence AATTCTAATACGACTCACTATAGGGAGAAGG [SEQ ID NO:62].

48. A probe/primer mixture for use in the detection of HPV type 52, type 58, type 67 or a combination thereof in a test sample, the mixture comprising an oligonucleotide primer set according to claim 44 or claim 45 and at least one oligonucleotide probe selected from the group consisting of:

oligonucleotide probes comprising one of the following nucleotide sequences:

5' CAACTGACCTAYWCTGCTATGA [SEQ ID NO:20]
5' GAAMCAACTGACCTAYWCTGCTAT [SEQ ID NO:21]

and oligonucleotide probes having one of the following structures:

5' X_2-CCAAGCAANCAACTGACCTAYWCTGCTAGCTTGG-X_3 [SEQ ID NO:22]

5' X_2-CCAAGCACTGACCTAYWCTGCTATGAGCTTGG-X_3 [SEQ ID NO:23]

wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

49. An oligonucleotide primer set for use in the detection of HPV type 33, type 58 or a combination thereof in a test sample comprising a first oligonucleotide primer comprising the sequence 5' X_1-CAAGTGTRACAACARGTTA [SEQ ID NO:24], wherein X_1 represents a nucleotide sequence comprising a promoter, and a second oliogonucleotide primer comprising the sequence 5' CAACTGACCTATWCTGCTA [SEQ ID NO:26].

50. An oligonucleotide primer set for use in the detection of HPV type 33, type 58 or a combination thereof in a test sample comprising a first oligonucleotide primer comprising the sequence 5' X_1-GCACAGSTAGGGCACACAA [SEQ ID NO:25], wherein X_1 represents a nucleotide sequence comprising a promoter, and a second oliogonucleotide primer comprising the sequence 5' ATCCTGAACCAACTGACCTA [SEQ ID NO:27].

51. An oligonucleotide primer set according to claim 49 or claim 50 wherein X_1 represents a nucleotide sequence comprising a T7 promoter.

52. An oligonucleotide primer set according to claim 51 wherein X_1 represents the nucleotide sequence AATTCTAATACGACTCACTATAGGGAGAAGG [SEQ ID NO:62].

53. A probe/primer mixture for use in the detection of HPV type 33, type 58 or a combination thereof in a test sample, the mixture comprising an oligonucleotide primer set according to claim 49 or claim 50 and at least one oligonucleotide probe selected from the group consisting of:

oligonucleotide probes comprising one of the following nucleotide sequences:

5' GTGACAGCTCAGAYGAGGATGA [SEQ ID NO:28]
5' GGCCAGATGGACAAGCACAAC [SEQ ID NO:29]

and oligonucleotide probes having one of the following structures:

5' X_2-CCAAGCGCCAGATGGACAAGCACAAGCTTGG-X_3 [SEQ ID NO:30]
5' X_2-CCGAGCGCCAGATGGACAAGCACAAGCTCGG-X_3 [SEQ ID NO:31]

wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

54. An oligonucleotide primer set for use in the detection of HPV type 45, type 18 or a combination thereof in a test sample comprising a first oligonucleotide primer comprising the sequence 5' X_1-AGCTCAATTCTGSCKTCA [SEQ ID NO:32], wherein X_1 represents a nucleotide sequence comprising a promoter, and a second oliogonucleotide primer comprising the sequence 5' ACGAGCAATTAAGCGAST [SEQ ID NO:35].

55. An oligonucleotide primer set for use in the detection of HPV type 45, type 18 or a combination thereof in a test sample comprising a first

oligonucleotide primer comprising the sequence 5' X_1-ACGGACACACAAAGGACA [SEQ ID NO:33], wherein X_1 represents a nucleotide sequence comprising a promoter, and a second oliogonucleotide primer comprising the sequence 5' ACGAGCAATTAAGCGAST [SEQ ID NO:35].

56. An oligonucleotide primer set for use in the detection of HPV type 45, type 18 or a combination thereof in a test sample comprising a first oligonucleotide primer comprising the sequence 5' X_1-GCACACCACGGACACACA [SEQ ID NO:34], wherein X_1 represents a nucleotide sequence comprising a promoter, and a second oliogonucleotide primer comprising the sequence 5' CCGACGAGCCGAACCACA [SEQ ID NO:36].

57. An oligonucleotide primer set according to any one of claims 54 to 56 wherein X_1 represents a nucleotide sequence comprising a T7 promoter.

58. An oligonucleotide primer set according to claim 57 wherein X_1 represents the nucleotide sequence AATTCTAATACGACTCACTATAGGGAGAAGG [SEQ ID NO:62].

59. A probe/primer mixture for use in the detection of HPV type 45, type 18 or a combination thereof in a test sample, the mixture comprising an oligonucleotide primer set according to claim 54 and at least one of: an oligonucleotide probe comprising the nucleotide sequence 5' AGCCCGACGAGCCGAACCACA [37] or an oligonucleotide probe having the structure 5' X_2-CCAAGCAGCCCGACGAGCCGAACCACAGCTTGG-X_3 [SEQ ID NO:39] wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in

close proximity.

60. A probe/primer mixture for use in the detection of HPV type 45, type 18 or a combination thereof in a test sample, the mixture comprising an oligonucleotide primer set according to claim 55 or claim 56 and at least one of: an oligonucleotide probe comprising the nucleotide sequence 5' GTTGTAAGTGTGAMGSCAGAATT [SEQ ID NO:38] or an oligonucleotide probe having the structure 5' X_2-CCAAGCAGCCCGACGAGCCGAACCACAGCTTGG-X_3 [SEQ ID NO:39] wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity.

61. A method of detecting HPV type 16, type 31, type 35 or a combination thereof in a test sample, the method comprising:

(a) assembling a reaction mixture comprising a primer cocktail as defined in any one of claims 13 to 16, an RNA directed DNA polymerase, a ribonuclease that hydrolyses the RNA strand of an RNA-DNA hybrid without hydrolysing single or double stranded RNA or DNA, an RNA polymerase that recognises said promoter, and ribonucleoside and deoxyribonucleoside triphosphates;

(b) incubating said reaction medium with a preparation of nucleic acid isolated from a test sample suspected of containing HPV under reaction conditions which permit a NASBA amplification reaction; and

(c) detecting and/or quantitatively measuring any HPV-specific product of the NASBA amplification reaction.

62. A method as claimed in claim 61 which comprises the further step of capturing the NASBA reaction product by hybridisation to a mixture of probe oligonucleotides attached to a solid support, wherein the mixure of probe oligonucleotides comprises oligonucleotides having the following sequences:

5' TGTAAWCATGCRTGGAGAWA [SEQ ID NO:7]
5' GAAACCSARSTGTAAWCATG [SEQ ID NO:8]
5' CATGCRTGGAGAWAYAMCTA [SEQ ID NO:9]
5' GACAGCTCAGAKGAGGAGGA [SEQ ID NO:10]
5' CAACTGAYCTMYACTGTTATGA [SEQ ID NO:11]
5' CAACTGAYCTMYACTGTTATGAGCAATT [SEQ ID NO:12]

63. A method as claimed in claim 61 wherein the reaction mixture further comprises probe oligonucleotides having the following structures:

5' X_2-CCGAGCACTGAYCTMYACTGTTATGAGCTCGG-X_3 [SEQ ID NO:13]
5' X_2-CCAAGCACTGAYCTMYACTGTTATGAGCTTGG-X_3 [SEQ ID NO:14]
5' X_2-CCAAGCCAACTGAYCTMYACTGTTATGAGCAGCTTGG-X_3 [SEQ ID NO:15]

wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity and the formation of any HPV-specific NASBA product in the NASBA reaction is monitored by detecting fluorescence from the fluorescent moiety.

64. A method of detecting HPV type 52, type 58, type 67 or a combination thereof in a test sample, the method comprising:

(a) assembling a reaction mixture comprising a primer cocktail as defined in any one of claims 19 to 22, an RNA directed DNA polymerase, a ribonuclease that hydrolyses the RNA strand of an RNA-DNA hybrid without hydrolysing single or double stranded RNA or DNA, an RNA polymerase that recognises said promoter, and ribonucleoside and deoxyribonucleoside triphosphates;

(b) incubating said reaction medium with a preparation of nucleic acid isolated from a test sample suspected of containing HPV under reaction conditions which permit a NASBA amplification reaction; and

(c) detecting and/or quantitatively measuring any HPV-specific product of the NASBA amplification reaction.

65. A method as claimed in claim 64 which comprises the further step of capturing the NASBA reaction product by hybridisation to a mixture of probe oligonucleotides attached to a solid support, wherein the mixure of probe oligonucleotides comprises oligonucleotides having the following sequences:

5' CAACTGACCTAYWCTGCTATGA [SEQ ID NO:20]
5' GAAMCAACTGACCTAYWCTGCTAT [SEQ ID NO:21]

66. A method as claimed in claim 64 wherein wherein the reaction mixture further comprises probe oligonucleotides having the following structures:

5' X_2-CCAAGCAANCAACTGACCTAYWCTGCTAGCTTGG-X_3 [SEQ ID NO:22]
5' X_2-CCAAGCACTGACCTAYWCTGCTATGAGCTTGG-X_3 [SEQ ID NO:23]

wherein X_2 and X_3 represent a fluorescent moiety and a

quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity and the formation of any HPV-specific NASBA product in the NASBA reaction is monitored by detecting fluorescence from the fluorescent moiety.

67. A method of detecting HPV type 33, type 58 or a combination thereof in a test sample, the method comprising:

(a) assembling a reaction mixture comprising a primer cocktail as defined in any one of claims 25 to 28, an RNA directed DNA polymerase, a ribonuclease that hydrolyses the RNA strand of an RNA-DNA hybrid without hydrolysing single or double stranded RNA or DNA, an RNA polymerase that recognises said promoter, and ribonucleoside and deoxyribonucleoside triphosphates;

(b) incubating said reaction medium with a preparation of nucleic acid isolated from a test sample suspected of containing HPV under reaction conditions which permit a NASBA amplification reaction; and

(c) detecting and/or quantitatively measuring any HPV-specific product of the NASBA amplification reaction.

68. A method as claimed in claim 67 which comprises the further step of capturing the NASBA reaction product by hybridisation to a mixture of probe oligonucleotides attached to a solid support, wherein the mixure of probe oligonucleotides comprises oligonucleotides having the following sequences:

5' GTGACAGCTCAGAYGAGGATGA [SEQ ID NO:28]

5' GGCCAGATGGACAAGCACAAC [SEQ ID NO:29]

69. A method as claimed in claim 67 wherein the reaction mixture further comprises probe oligonucleotides having the following structures:

5' X_2-CCAAGCGCCAGATGGACAAGCACAAGCTTGG-X_3 [SEQ ID NO:30]
5' X_2-CCGAGCGCCAGATGGACAAGCACAAGCTCGG-X_3 [SEQ ID NO:31]

wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity and the formation of any HPV-specific NASBA product in the NASBA reaction is monitored by detecting fluorescence from the fluorescent moiety.

70. A method of detecting HPV type 18, type 45 or a combination thereof in a test sample, the method comprising:

(a) assembling a reaction mixture comprising a primer cocktail as defined in any one of claims 31 to 34, an RNA directed DNA polymerase, a ribonuclease that hydrolyses the RNA strand of an RNA-DNA hybrid without hydrolysing single or double stranded RNA or DNA, an RNA polymerase that recognises said promoter, and ribonucleoside and deoxyribonucleoside triphosphates;

(b) incubating said reaction medium with a preparation of nucleic acid isolated from a test sample suspected of containing HPV under reaction conditions which permit a NASBA amplification reaction; and

(c) detecting and/or quantitatively measuring any HPV-specific product of the NASBA amplification reaction.

71. A method as claimed in claim 70 which

comprises the further step of capturing the NASBA reaction product by hybridisation to a mixture of probe oligonucleotides attached to a solid support, wherein the mixure of probe oligonucleotides comprises oligonucleotides having the following sequences:

5' AGCCCGACGAGCCGAACCACA [SEQ ID NO:37]
5' GTTGTAAGTGTGAMGSCAGAATT [SEQ ID NO:38]

72. A method as claimed in claim 71 wherein the reaction mixture further comprises a probe oligonucleotide having the following sequence:

5' X_2-CCAAGCAGCCCGACGAGCCGAACCACAGCTTGG-X_3 [SEQ ID NO:39]

wherein X_2 and X_3 represent a fluorescent moiety and a quencher moiety capable of substantially or completely quenching the fluorescence from the fluorescent moiety when the two are held together in close proximity and the formation of any HPV-specific NASBA product in the NASBA reaction is monitored by detecting fluorescence from the fluorescent moiety.

73. A reagent kit for use in the detection of HPV type 16, type 31, type 35 or a combination thereof by NASBA, the kit comprising an oligonucleotide primer cocktail as defined in any one of claims 13 to 16 and optionally an enzyme mixture comprising an RNA directed DNA polymerase, a ribonuclease that hydrolyses the RNA strand of an RNA-DNA hybrid without hydrolysing single or double stranded RNA or DNA, an RNA polymerase that recognises said promoter.

74. A reagent kit for use in the detection of HPV type 52, type 58, type 67 or a combination thereof by NASBA, the kit comprising an oligonucleotide primer

cocktail as defined in any one of claims 19 to 22 and optionally an enzyme mixture comprising an RNA directed DNA polymerase, a ribonuclease that hydrolyses the RNA strand of an RNA-DNA hybrid without hydrolysing single or double stranded RNA or DNA, an RNA polymerase that recognises said promoter.

75. A reagent kit for use in the detection of HPV type 33, type 58 or a combination thereof by NASBA, the kit comprising an oligonucleotide primer cocktail as defined in any one of claims 25 to 38 and optionally an enzyme mixture comprising an RNA directed DNA polymerase, a ribonuclease that hydrolyses the RNA strand of an RNA-DNA hybrid without hydrolysing single or double stranded RNA or DNA, an RNA polymerase that recognises said promoter.

76. A reagent kit for use in the detection of HPV type 18, type 45 or a combination thereof by NASBA, the kit comprising an oligonucleotide primer cocktail as defined in any one of claims 31 to 34 and optionally an enzyme mixture comprising an RNA directed DNA polymerase, a ribonuclease that hydrolyses the RNA strand of an RNA-DNA hybrid without hydrolysing single or double stranded RNA or DNA, an RNA polymerase that recognises said promoter.

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